COMBINATION TREATMENT OF CANCER USING VACCINE AND A CHEMOTHERAPEUTIC DRUG

The present invention relates to the treatment of cancer and in particular to treatments based on a combination of chemotherapy and immunotherapy.

Cytotoxic T lymphocytes (CTL) directed at tumour cells presenting unique peptide on MHC class I molecules constitute a potentially powerful effector arm of host immunity to tumours. Immunisation strategies that aim to induce or restimulate tumour-specific CTL may therefore provide an effective and practical approach to cancer treatment (Rosenberg, *Nature* 2001; **411**:380-4). While the utility of such immunotherapy has been shown in many preclinical studies, it is unlikely that this treatment modality alone will be sufficient to cure patients with significant metastatic disease, and hence high tumour burdens. This is because tumours may mutate such that they no longer present the unique peptide. In fact, immunotherapy may hold most promise as an adjunct to conventional strategies aimed at cytoreduction, such as chemotherapy (Greenberg, *Adv Immunol* 1991; **49**:281-355).

However, it is well recognised that many chemotherapeutic drugs actively suppress cell-mediated immunity. Traditional chemotherapeutic drug dosing schedules involving maximum tolerated doses (MTD) are also associated with considerable toxicity and discomfort to cancer patients, requiring extended rest periods to allow recovery. In light of recent studies describing the potent anti-angiogenic capacity of low drug doses administered in a metronomic fashion (Browder *et al, Cancer Res* 2000; **60**:1878-86; Klement *et al, J Clin Invest* 2000; **105**:R15-24), it is now likely that low dose regimes will find favour in the clinical setting.

The present inventors have assessed the efficacy of anti-tumour responses generated by a combination of metronomic dosing of cyclophosphamide (CTX) with an immunisation strategy aimed at eliciting tumour-specific CTL. A mouse tumour model of B16 melanoma modified to express a defined CTL epitope, and an immunisation protocol involving injection of recombinant vectors encoding this CTL epitope was used. They have found that the combination of metronomic

administration of CTX with immunisation induces anti-tumour responses that are dramatically enhanced over immunotherapy or chemotherapy alone, and that are also enhanced over immunotherapy combined with CTX administered in a MTD. In addition, they have shown that a combination of the immunisation protocol described above and vinblastine is more effective than either immunisation or vinblastine alone, and that a combination of metronomic administration of CTX and immunotherapy administered by dendritic cells loaded with tumour-specific peptide is significantly more effective that either of these therapies alone.

Accordingly, in a first aspect, the present invention provides the use of an agent for stimulating an immune response against a cancer, and a chemotherapeutic drug in the manufacture of a medicament for the treatment of said cancer wherein said drug is for administration in a metronomic schedule.

In a second aspect, the invention provides a product containing an agent for stimulating an immune response against a cancer; and a chemotherapeutic drug as a combined preparation for simultaneous, sequential or separate use in treating said cancer, the drug being for administration in a metronomic schedule.

In a third aspect, the invention provides a method of treating a patient suffering from cancer, comprising administering to the patient:

an agent for stimulating an immune response against said cancer; and

a chemotherapeutic drug;

wherein said drug is administered in a metronomic schedule.

The present invention is based on the finding that, although chemotherapeutic anti-tumour drugs administered using the MTD regime might prevent or severely retard an immune response, such drugs administered in an metronomic schedule retain an anti-tumour effect but do not have such severe immune suppression effects. Accordingly, they can be administered in combination with treatments which cause an immune response to be raised against the tumour, resulting in an unexpectedly effective anti-tumour therapy.

As used in conjunction with the present invention, a "metronomic schedule" means the administration of the chemotherapeutic drug at doses lower than the MTD which are nevertheless effective to prevent or delay growth of the cancer, preferably at frequent intervals and without extended rest periods. Over a given treatment cycle, the metronomic schedule may be such that 80% or less (and preferably 70%, 60%, 50%, 40%, 30%, 20% or less) of the drug is administered than would have been administered using the MTD regime. The drug may be administered in a range from continuous fusion to weekly or 1-3 times a week. Such a schedule is to be contrasted with the maximum tolerated dose (MTD) regime normally used for chemotherapeutic drugs, where administration may consist of high dose(s) administered over short periods followed by a period (often 2-3 weeks) where no drug is administered in order to allow recovery of non-cancer cells which are killed by the high doses.

The metronomic schedule may be such as to allow the drug to be anti-angiogenic, i.e. to cause apoptosis of vascular endothelial cells within the tumour, rather than to act directly on the tumour cells. Because the direct action of the drug on cancer cells is reduced, concomitant selection of drug resistant tumour cells is also avoided. The anti-angiogenic effect of the schedule can be measured as described in Browder *et al, Cancer Res* 2000; **60**:1878-86.

The chemotherapeutic drug may be a small molecule cytotoxic agent, i.e. a compound with the ability to kill mammalian cells having a molecular weight of, for example, less than 700 daltons. Such compounds may contain toxic metals capable of having a cytotoxic effect. Furthermore, it is to be understood that these small molecule cytotoxic agents also include pro-drugs, i.e. compounds that decay or are converted under physiological conditions to release cytotoxic agents.

One suitable drug useful in the invention is cyclophosphamide (CTX), which is an alkylating agent commonly used in chemotherapy. CTX is known to have immuno-suppressive qualities, and indeed is commonly used as a suppressant in autoimmune conditions such as arthritis and lupus nephritis. It has also been shown to have

immune-potentiating activity in some settings (Askenase *et al, J Exp Med* 1975; **141**:697-702; Maguire *et al, J Invest Dermatol* 1967; **48**:39-43; Mitsuoka *et al, Nature* 1976; **262**:77-8, Ehrke *et al, Semin Oncol* 1989; **16**:230-53). CTX has also been administered in combination with immunotherapy (Machiels *et al, Cancer Res.*, 2001; **61**: 3689-3697; Vierboom *et al, Int. J. Cancer,* 2000; **87**:253-260) and been shown to enhance such immunotherapy in certain circumstances. However, in neither of these studies was CTX administered in a metronomic schedule, being administered in a single dose before immunotherapy. In addition, Vierboom *et al* were not able to show any enhancement of immunotherapy when CTX was administered at a low dose. Therefore, prior to the present invention, it was not clear or predictable what effect CTX would have on the immune system when administered in a metronomic schedule. The present inventors have found that the combination of CTX when administered in a metronomic schedule and immunotherapy is unexpectedly efficacious in killing tumour cells.

Metronomic dosing of CTX may comprise 130-250, 150-200, 160-180, 170-175 or 175 mg/kg of CTX injected or otherwise administered every 1-8, 3-7, 5 or 6 days (as compared to a typical MTD dosing of a 21 day cycle of 150 mg/kg CTX administered every other day over 6 days (i.e. three injections) followed by 15 days rest), or may be as described in Browder *et al, Cancer Res* 2000; **60**:1878-86. CTX may alternatively be administered as described in Man *et al, Cancer Res.* 2002 **62**:2731-2735, i.e. continuously in drinking water at a dose of 10-40 mg/kg/day. This metronomic schedule sustains cytolytic activity against proliferating endothelial cells required for blood vessel growth, thereby starving developing tumours of oxygen.

Another drug suitable for use in the invention is vinblastine, which may be administered at 0.5-5, 0.75-2, 0.85-1.5 or 1 mg/kg once, twice, three, four, five times or more every week, or as described in Klement *et al, J Clin Invest* 2000; **105**:R15-24.

Further drugs suitable for use in the invention include 5-fluorocil, 6-mercaptopurine, doxorubicin, taxol, cisplatinum, etopside, carboplatin, paclitaxel and topotecan. 5-fluorocil and 6-mercaptopurine may be administered as a continuous fusion

(preferably as described in Browder *et al, Cancer Res* 2000; **60**:1878-86). Taxol and cisplatinum may be administered as described in Klement *et al, Cancer Res.* 2002; **8**:221-232, etopside and carboplatin may be administered as described in Bello *et al, Cancer Res,* 2001; **61**: 7501-7506 and topotecan may be administered as described in Soffer *et al, J. Pediatr. Surg,* 2001; **36**:1781-1784.

The agent for stimulating an immune response against said cancer may be a tumour-specific antigen, preferably recognised by cytotoxic T cells (although antigens recognised by antibodies and such are included within the present invention), and/or nucleic acid encoding such an antigen. Examples include melanoma epitopes (e.g. MAGE-1 HLA-A1 restricted epitope) and other cancer-specific epitopes (e.g. the renal cell carcinoma associated antigen G250 restricted by HLA-A2). Further cancer-associated antigens are listed in the HLA Factbook (Barclay (Ed) Academic Press), and many others have been and are being identified.

The inventors have shown that treatment in accordance with the present invention allows memory T cells (i.e. those with restimulatory capacity) to survive. This means that cycles of the treatment can be repeated with the immunotherapeutic aspect still being effective by virtue of the re-stimulation of T cells activated by previous immunotherapy. In addition, this effect can be exploited to counter the possibility of the tumour losing the ability to express a certain antigen and hence be affected by the immunotherapy. Thus, in certain embodiments, the agent for stimulating an immune response may comprise a plurality of different tumour-specific antigens and/or nucleic acid encoding such antigens. After the initial treatment with such an agent and the chemotherapeutic drug, subsequent rounds of treatment may involve the re-stimulation of T cells recognising one or a combination of the plurality of antigens. In such embodiments, it is preferred if there is a delay between administration of the agent and the drug.

The antigen may be administered by means of dendritic cells loaded with the antigen (Mayordomo *et al. Nat Med.* 1995 Dec; **1**(12):1297-302; Hsu *et al. Nat Med.* 1996 Jan; **2**(1):52-8). Other methods for administration of such antigens are known to

those of skill in the art and include administration of the naked peptide, of liposomes containing the peptide, etc.

The agent for stimulating an immune response against said cancer may be a T cell specific for the tumour, which may be administered by adoptive transfer (Cheever *et al, J Exp Med* 1986 May 1; **163**(5):1100-12; Vierboom *et al, Int J. Cancer*, 2000; **87**:253-260; Greenberg, *J Immunol.* 1986 Mar 1; **136**(5):1917-22.).

Nucleic acid encoding a tumour specific antigen, which may be RNA or DNA, may be administered in a suitable vector. In a preferred embodiment, two different vectors are used to administer the nucleic acid, thereby focussing immune responses to the recombinant antigen shared by the vectors. One of the vectors may be a replication-impaired pox virus, such as modified vaccinia virus (MVA). In this instance, it is preferred if the replication-impaired pox virus is administered to boost the immune response to a previously-administered DNA vector (Hanke *et al, Vaccine* 1999; **17**:589-96; Schneider *et al, Nat Med* 1998; **4**:397-402; Kent *et al, J Virol* 1998; **72**:10180-8).

Nucleic acid may be administered using various types of recombinant viruses as vehicles for DNA inoculation or by techniques that use "naked" DNA. The tumour-specific antigen may be administered such that it is expressed in the subject to be treated for example in the form of a recombinant DNA molecule comprising a polynucleotide encoding the antigen operatively linked to a nucleic acid sequence which controls expression, such as in an expression vector. Such a vector will thus include appropriate transcriptional control signals including a promoter region capable of expressing the coding sequence, said promoter being operable in the subject to be treated. Thus, for human therapy, the promoter, which term includes not only the sequence necessary to direct RNA polymerase to the transcriptional start site, but also, if appropriate, other operating or controlling sequences including enhancers, is preferably a human promoter sequence from a human gene, or from a gene which is typically expressed in humans, such as the promoter from human cytomegalovirus (CMV). Among known eukaryotic promoters suitable in this regard are the CMV

immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter. A polynucleotide sequence and transcriptional control sequence may be provided cloned into a replicable plasmid vector, based on commercially available plasmids, such as pBR322, or may be constructed from available plasmids by routine application of well known, published procedures. The expression vectors may also include selectable markers, such as for antibiotic resistance, which enable the vectors to be propagated.

Expression vectors capable *in situ* of synthesising antigen may be introduced directly by physical methods. Examples of these include topical application of the 'naked' nucleic acid vector in an appropriate vehicle for example in solution in a pharmaceutically acceptable excipient such as phosphate buffered saline (PBS). Other physical methods of administering the DNA directly to the recipient include ultrasound, electrical stimulation, electroporation and microseeding.

Generally, vectors for expressing antigen for use in the invention comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host. In certain embodiments in this regard, the vectors provide for specific expression. Such specific expression may be inducible expression or expression only in certain types of cells or both inducible and cell-specific.

A great variety of expression vectors can be used to express antigen for use in the invention. Such vectors include, among others, chromosomal, episomal and virus-derived vectors, e.g., vectors derived from bacterial plasmids, from bacteriophage, from transposons, from yeast episomes, from insertion elements, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived

from plasmid and bacteriophage genetic elements, such as cosmids and phagemids. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The appropriate DNA sequence may be inserted into the vector by any of a variety of well-known and routine techniques, such as, for example, those set forth in Sambrook et al., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989).

It is to be noted that CTL-mediated immune response to tumours can be initiated without prior knowledge of defined tumour antigens. Thus, the agent for stimulating an immune response need not be a tumour-specific antigen. Instead, irradiated or lysed tumour cells (e.g. from biopsy tissue), tumour cells fused with dendritic cells, and/or heatshock proteins isolated from tumour tissue may be used. Tumour tissue can also be modified to express gene sequences that enhance immune responses, (e.g. cytokine genes) before being irradiated and injected.

In the present invention, the agent for stimulating an immune response is preferably administered before the drug, although it is to be understood that the present invention includes treatments when the drug is administered before the agent for stimulating an immune response and when the respective treatments are administered simultaneously.

The time, if any, between the respective treatments may be varied widely. It is preferred if the drug is delivered after the peak CTL induction. The drug may be administered from 3-100 days after the agent and preferably 10-30, 14-30 or 15-18 days after the agent.

In addition to the agent for stimulating an immune response against said cancer and the chemotherapeutic drug, an angiogenesis inhibitor may be administered. Suitable inhibitors are known to those of skill in the art and include TNP-470.

In further aspects, the present invention provides

a) a method of treating a patient suffering from cancer, comprising administering to the patient:

an agent for stimulating an immune response against said cancer; and

a drug effective in preventing or delaying angiogenesis;

b) the use of an agent for stimulating an immune response against a cancer, and a drug effective in preventing or delaying angiogenesis in the manufacture of a medicament for the treatment of said cancer; and

c) a product containing an agent for stimulating an immune response against a cancer; and a drug effective in preventing or delaying angiogenesis as a combined preparation for simultaneous, sequential or separate use in treating said cancer.

Medicaments in accordance with the invention will usually be supplied as part of a sterile, pharmaceutical composition which will normally include a pharmaceutically acceptable carrier. This pharmaceutical composition may be in any suitable form, (depending upon the desired method of administering it to a patient). It may be provided in unit dosage form, will generally be provided in a sealed container and may be provided as part of a kit. Such a kit would normally (although not necessarily) include instructions for use. It may include a plurality of said unit dosage forms.

The pharmaceutical composition may be adapted for administration by any appropriate route, for example by the oral (including buccal or sublingual), rectal, nasal, topical (including buccal, sublingual or transdermal), vaginal or parenteral (including subcutaneous, intramuscular, intravenous or intradermal) route. Such compositions may be prepared by any method known in the art of pharmacy, for example by admixing the active ingredient with the carrier(s) or excipient(s) under sterile conditions.

Pharmaceutical compositions adapted for oral administration may be presented as discrete units such as capsules or tablets; as powders or granules; as solutions, syrups or suspensions (in aqueous or non-aqueous liquids; or as edible foams or whips; or as emulsions).

Suitable excipients for tablets or hard gelatine capsules include lactose, maize starch or derivatives thereof, stearic acid or salts thereof. Suitable excipients for use with soft gelatine capsules include for example vegetable oils, waxes, fats, semi-solid, or liquid polyols etc.

For the preparation of solutions and syrups, excipients which may be used include for example water, polyols and sugars. For the preparation of suspensions oils (e.g. vegetable oils) may be used to provide oil-in-water or water in oil suspensions.

Pharmaceutical compositions adapted for transdermal administration may be presented as discrete patches intended to remain in intimate contact with the epidermis of the recipient for a prolonged period of time. For example, the active ingredient may be delivered from the patch by iontophoresis as generally described in *Pharmaceutical Research*, 3(6):318 (1986).

Pharmaceutical compositions adapted for topical administration may be formulated as ointments, creams, suspensions, lotions, powders, solutions, pastes, gels, sprays, aerosols or oils. For infections of the eye or other external tissues, for example mouth and skin, the compositions are preferably applied as a topical ointment or cream. When formulated in an ointment, the active ingredient may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the active ingredient may be formulated in a cream with an oil-in-water cream base or a water-in-oil base. Pharmaceutical compositions adapted for topical administration to the eye include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent. Pharmaceutical compositions adapted for topical administration in the mouth include lozenges, pastilles and mouth washes.

Pharmaceutical compositions adapted for rectal administration may be presented as suppositories or enemas.

Pharmaceutical compositions adapted for nasal administration wherein the carrier is a solid include a coarse powder having a particle size for example in the range 20 to 500 microns which is administered in the manner in which snuff is taken, i.e. by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable compositions wherein the carrier is a liquid, for administration as a nasal spray or as nasal drops, include aqueous or oil solutions of the active ingredient.

Pharmaceutical compositions adapted for administration by inhalation include fine particle dusts or mists which may be generated by means of various types of metered dose pressurised aerosols, nebulizers or insufflators.

Pharmaceutical compositions adapted for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations.

Pharmaceutical compositions adapted for parenteral administration include aqueous and non-aqueous sterile injection solution which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation substantially isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. Excipients which may be used for injectable solutions include water, alcohols, polyols, glycerine and vegetable oils, for example. The compositions may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carried, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets.

The pharmaceutical compositions may contain preserving agents, solubilising agents, stabilising agents, wetting agents, emulsifiers, sweeteners, colourants, odourants, salts (substances of the present invention may themselves be provided in the form of a pharmaceutically acceptable salt), buffers, coating agents or antioxidants. They may also contain therapeutically active agents in addition to the substance of the present invention.

Preferred features of each aspect of the invention are as for each of the other aspects *mutatis mutandis*. The prior art documents mentioned herein are incorporated to the fullest extent permitted by law.

Examples

The invention will now be described in the following non-limiting examples. Reference is made to the accompanying drawings in which:

Figure 1 illustrates that the sequential immunisation with recombinant vectors encoding a tumour-specific antigen induces resistance to tumour challenge. CTL responses in C57BL/6 mice were primed by i.m. injection of DNAmel3, and then boosted by i.v. injection with MVAmel3 14 days later. **A)** Representative FACS plots showing expansion of a population of Flu-$NP_{366-374}/D^b$ tetramer$^+$ $CD8^+$ cells in the blood in response to this immunisation regime are presented. Percentages of cells in the upper quadrants are indicated. **B)** Immunised animals were challenged with 3 x 10^5 B16mel3 cells 7 days after MVA boost, and tumour progression was compared with growth observed in unimmunised animals. Mean tumour size per group ($n = 5$) ± S.E. are shown.

Figure 2 illustrates that combining immunotherapy with metronomic dosing of CTX provides prolonged resistance to tumour challenge. **A)** Progression of B16 tumours was monitored in animals that were subjected to one of the following treatment schedules: ○, no treatment; ●, immunisation prior to challenge as outlined in Figure 1; ■, treatment with CTX administered as MTD (repeated cycles of 150 mg/kg every other day for 6 days followed by 15 day rest); ◆, metronomic treatment with CTX (175 mg/kg every 6 days); Δ, a combination of prior immunisation and CTX by MTD; ◊, a combination of prior immunisation and metronomic CTX. Arrows indicate CTX dosing. Mean tumor size per group ($n = 5$) ± S.E. are shown. **B)** Blood was taken from immunised animals two days before tumour challenge and 15 and 28 days after challenge in order to examine the effects of CTX treatment on levels of Flu-$NP_{366-374}$-

specific CTL, as measured by FACS analysis with Flu-$NP_{366-374}/D^b$ tetramers. Representative FACS plots are shown, with the percentages of cells in the upper quadrants indicated. **C)** A repeat of experiment shown in A without groups treated with CTX by MTD, and with metronomic CTX treatment initiated on the day of tumour challenge.

Figure 3 illustrates that activated CTL with a $CD43^{lo}$ phenotype are resistant to metronomic CTX-mediated deletion, and retain potent restimulatory capacity. **A)** CTL responses were initiated by immunization as in Figure 1, and then blood was taken for FACS analysis on the indicated days. CD43 expression was assessed on gated $CD8^+$ Flu-$NP_{366-374}/D^b$ $tetramer^+$ cells. **B)** CTL responses were monitored in the blood of immunized animals following metronomic CTX treatment initiated on the days indicated. The total number $CD8^+$ Flu-$NP_{366-374}/D^b$ $tetramer^+$ cells per mm^3 of blood was extrapolated from white blood cell counts. Data are presented as mean number of $CD8^+$ Flu-$NP_{366-374}/D^b$ $tetramer^+$ cells per treatment group ($n = 5$) ± S.E. The inset in the lower panel provides a magnification of scale. **C)** Each of the treatment groups in B was injected with Vaccmel3 infected syngeneic splenocytes in order to restimulate Flu-$NP_{366-374}$-specifc CTL. The mean number of $CD8^+$ Flu-$NP_{366-374}/D^b$ $tetramer^+$ cells per group prior to injection (pre-), and 7 days after injection (post-) is shown.

Figure 4 illustrates that combining immunotherapy with metronomic dosing of vinblastine provides prolonged resistance to tumour challenge. Progression of B16 tumours was monitored in animals that were subjected to one of the following treatment schedules: ○, no treatment; ●, immunisation prior to challenge as outlined in Figure 1; ◊, metronomic treatment with vinblastine (1 mg/kg twice weekly); ◆, a combination of prior immunisation and metronomic vinblastine.

Figure 5 illustrates that combining immunotherapy by way of dendritic cells dosed with tumour-specific antigen with metronomic dosing of CTX provides prolonged resistance to tumour challenge. Progression of B16 tumours was monitored in animals that were subjected to one of the following treatment schedules: ○, dendritic

cells only; ●, dendritic cells only and CTX; ■, dendritic cells and tumour-specific antigen; ◊, a combination of dendritic cells and tumour-specific antigen and metronomic CTX (175 mg/kg every 6 days).

Materials and Methods

Mice. C57BL/6 mice were from breeding pairs originally obtained from Jackson Laboratories, Bar Harbor, Maine. All mice were maintained at the Biomedical Services Unit of John Radcliffe Hospital by brother x sister mating; *in vivo* experimental protocols were performed according to institutional guidelines.

In vitro culture media and reagents. All cultures were maintained in complete medium (CM) comprising of RPMI (Sigma-Aldrich , Dorset, England) with 2 mM glutamine, 1 % penicillin-streptomycin, 5 x 10^{-5} M 2-mercapto-ethanol (all Invitrogen Ltd, Paisley, UK) and 10 % fetal bovine serum (Globepharm, Guildford, England).

Immunisation strategy. Animals were primed for $NP_{366-374}$-specific CTL responses by intramuscular injection of 50 μg of plasmid DNA encoding the *mel3* polyepitope (DNA-mel3). The mel3 construct consists of a string of five HLA-A2 and two HLA-A1 melanoma epitopes, and an influenza nucleoprotein epitope restricted by H-2D^b (Palmowski *et al, J Immunol* 2002; **168**:4391-8). Only the influenza epitope $NP_{366-374}$ (ASNENMDAM) is presented on a C57BL/6 background. Mice were boosted 14-60 days after DNA immunization by i.v. injection with 10^6 PFU of recombinant MVA encoding the *mel3* polyepitope construct (MVA-mel3). In some instances, animals were boosted a second time by i.v injection with 1 x 10^6 syngeneic splenocytes that had been infected with recombinant vaccinia encoding the *mel3* polyepitope construct (Vacc-mel3). Splenocytes (5 x 10^7 cells/ml) were infected with 10^8 PFU of Vacc-mel3 in PBS supplemented with 0.1 % BSA (Sigma-Aldrich) at 37°C for 2 h, washed, and injected i.v. into the lateral tail vein.

Cyclophosphamide dosing schedule. CTX (ASTA Medica Ltd, Cambridge, UK) was reconstituted in sterile distilled H_2O and administered by i.p. injection. The dosing schedules of Browder *et al, Cancer Res* 2000; **60**:1878-86 were adopted for these studies. Thus metronomic dosing consisted of 175 mg/kg of CTX injected every 6

days, and standard dosing consisted of a 21 day cycle of 150 mg/kg CTX administered every other day over 6 days (i.e. three injections) followed by 15 days rest.

Vinblastine dosing schedule. Vinblastine sulphate (David Bull Laboroatories, Warwick, UK) was administered by i.p injection twice weekly at 1 mg/kg.

Tumour immunity assay. Groups of mice ($n = 5$) were challenged with the tumour cell line B16-mel3, a derivative of the B16.F10 melanoma cell line (C57BL/6, H-2^b) that had been modified to express the *mel3* polyepitope construct in combination with enhanced green fluorescent protein from a bicistronic mRNA (pIRES2-EGFP vector, Clontech, Basingstoke, UK). Challenge was with 3 x 10^5 B16-mel3 tumor cells injected s.c. into the left flank. Mice were monitored for tumour growth every 3-4 days, and tumour size for each group was calculated as the mean of the products of bisecting diameters (± SE.). Measurements were terminated for each group when the first animal developed a tumour in excess of 200 mm^2.

Monitoring CTL responses with MHC tetramers. Tetrameric H-2 D^b/ $NP_{366-374}$ peptide complexes were prepared as outlined in Altman *et al, Science* 1996; **274**:94-6, and used to stain fresh PBL isolated from the lateral tail vein. Approximately 5 x 10^5 PBL were suspended in 20 μl CM and incubated with 0.5 μg of tetramer complexes at 37 °C for 20 min. The cells were then incubated with anti-CD8α and anti-CD43 (BD Pharmingen, San Diego, CA) for 10 min at 4 °C, washed twice with PBS, and resuspended in PBS for FACS analysis. Cells were analysed with FACScan hardware and CellQuest software (BD Biosciences , Mountain View, CA). Blood was also collected to perform white blood cell counts so that total lymphocyte numbers per mm^3 of blood could be extrapolated. White blood cell counts were performed using a Micros60 blood counter (ABX–PARC Euromedecine, France).

Culture of bone marrow-derived dendritic cells (DC). Bone marrow cells from C57BL/6 mice were cultured in CM containing 20 ng/ml IL-4 and 20 ng/ml GM-CSF for 7 d. Cultures typically contained 70-100 % DC as determined by fluorescent staining with the anti-CD11c antibody N418. DC were loaded with peptide antigen by incubation in CM containing 10 μM synthetic peptide $LCMV_{GP33}$ (KAVYNFATM) for 2 h. The DC were extensively washed to remove free peptide and then injected subcutaneously (1 x 10^5 cells, each flank).

Tumour cell line. B16-GP33 is a derivative of the B16 (C57BL/6, H-2^b) which has been modified to express a minigene encoding $LCMV_{33-41}$ (Prevost-Blondel *et al. J Immunol.* 1998 **161**(5): 2187-94).

Statistical analysis. The statistical significance of differential findings between experimental groups was determined by the Student's t test. Findings were regarded as significant if two-tailed *P* values were < 0.05.

Example 1- Sequential immunisation of C57BL/6 mice with DNA and vaccinia vectors encoding a tumour-specific antigen can elicit CTL responses capable of resisting tumour challenge

A murine model of melanoma expressing a unique CTL epitope was used to assess specific immune responses to tumours *in vivo*. It has previously been shown that an immunisation strategy involving sequential injection of recombinant DNA and vaccinia vectors encoding a specific epitope can elicit powerful CTL-mediated immune responses (Hanke *et al, Vaccine* 1999; **17**:589-96; Schneider *et al, Nat Med* 1998; **4**:397-402; Kent *et al, J Virol* 1998; **72**:10180-8). Immunisation with vectors encoding the polyepitope construct '*mel3*' can be used to initiate CTL responses in both H-2^b mice and HLA-A2-transgenic mice (Palmowski *et al, J Immunol* 2002; **168**:4391-8). In H-2^b strains, such as C57BL/6, CTL responses are induced solely to the D^b-restricted influenza nucleoprotein epitope ($NP_{366-374}$). The efficacy of sequential immunisation with DNA and vaccinia (Modified Vaccinia Ankara strain, MVA) (Carroll *et al,* Vaccine 1997; **15**:387-94) vectors encoding *mel3* to induce $NP_{366-374}$-specific responses capable of resisting challenge with B16 melanoma cells also modified to express *mel3* was assessed. DNA (DNA-mel3) was injected intramuscularly to prime $NP_{366-374}$-specific CTL, and then these responses were boosted several days later by i.v. injection of recombinant MVA (MVA-mel3). Proliferation of specific CTL was monitored in the blood using H-2 Db/$NP_{366-374}$ tetramers and FACS analysis. In a typical experiment shown in Fig. 1 *A*, DNA-mel3 alone induced activation and proliferation of $NP_{366-374}$-specific CTL such that, by day 13, post-injection the tetramer positive population represented an average of 1.5 ± 0.21 % of circulating $CD8^+$ lymphocytes ($n = 5$). When MVA-mel3 was injected intravenously 14 days after DNA immunisation, the $NP_{366-374}$-specific cells were

effectively boosted, proliferating to an average of 25.6 ± 4.5 % of $CD8^+$ lymphocytes 7 days later. For the tumour challenge experiment shown in Fig. 1 *B*, B16-mel3 melanoma cells were injected subcutaneously 7 days after MVA-mel3 boost. These cells had been transfected with the *mel3* polyepitope construct together with a green fluorescent protein (GFP) sequence to monitor antigen expression. Anti-tumour responses were observed in all immunised animals, with engraftment and growth of the administered tumours retarded by 9 days relative to growth in control animals (Fig. 1B). Analysis of GFP expression in tumours that eventually developed in the immunised animals indicated selective loss of antigen expression (not shown). These data indicate that this immunisation strategy can induce stimulation of significant anti-tumour responses, but immunological escape occurs by antigen loss.

Example 2 – Metronomic dosing with cyclophosphamide enhances anti-tumour effects of CTL-mediated immunity.

The immunotherapy model described in Example 1 was utilised to investigate the feasibility of combining CTX treatment with anti-tumour immunotherapy (Fig. 2 *A*). CTX treatment alone, whether delivered by a standard MTD regimen or a metronomic low dose schedule, induced anti-tumour activity resulting in retarded tumour growth relative to untreated controls. Measurements were terminated for each group when the first animal developed a tumour in excess of 200 mm^2. Thus, while measurements were terminated for the untreated group at day 21 after challenge, animals undergoing the MTD regime survived until day 34 after tumour challenge, and animals receiving the metronomic schedule until day 42 after challenge. The slower tumour growth observed in animals treated with the metronomic regime supports previous studies showing that this schedule induces potent anti-tumour responses, most likely by an anti-angiogenic mechanism (Browder *et al, Cancer Res* 2000; **60**:1878-86; Klement *et al, J Clin Invest* 2000; **105**:R15-24).

When either of these dosing regimes was administered together with immunotherapy, the combination treatments proved to be more potent than any treatment modality alone. Significantly, the combination of immunotherapy and metronomic dosing of CTX was the most successful of all of the treatment regimes. There was significantly

delayed tumour progression in this group, with the first animal bearing a tumour in excess of 200 mm^2 sacrificed at 81 days after challenge (versus 48 days in the immunotherapy/MTD group). Two out of 5 animals were still tumour free at this point, and remained tumour free in excess of 100 days post-challenge in the absence of further CTX treatment, until they too succumbed to tumour. In contrast, all animals in the other groups were bearing tumours at the time measurements were terminated and animals were sacrificed.

FACS analysis of blood from all immunised animals indicated that CTX treatment administered by either dosing regime caused generalised cytopenia including loss of $NP_{366-374}$-specific CTL (Fig 2 *B*). However, CTX-induced loss of $NP_{366-374}$-specific CTL was not as severe soon after tumour challenge (day 15) in animals that received metronomic dosing as opposed to the MTD regime. Thus, by avoiding some of the toxicity associated with the MTD schedule, the metronomic low dose schedule permits greater CTL activity, and hence provides a more efficacious combination with immunotherapy.

Example 3 – Effects of timing of CTX therapy on anti-tumour response.

In Example 2, CTX treatment (by either dosing regime) was initiated 10 days after tumour challenge, which was 17 days after MVA boost. Interestingly, when low dose metronomic therapy was initiated on the day of tumour challenge (Fig 2 C), the combination therapy was not as potent as in the earlier experiment, although the combination therapy was still greatly enhanced over the other treatment groups. In this experiment, the metronomic therapy had been initiated at a time when the CTL proliferation was likely to be peaking after MVA boost. Thus, these results imply that the toxicity associated with CTX treatment, even at the low doses used, still has some detrimental activity on proliferating CTL, thereby weakening the anti-tumour responses. Therefore, while the combination of immunotherapy and metronomic dosing of CTX provides a powerful treatment strategy, prudent timing of chemotherapy relative to immunotherapy may be required to provide the most effective response.

Example 4 - Metronomic dosing with cyclophosphamide reduces numbers of proliferating 'effector' CTL but spares CTL with restimulatory capacity.

The effect of CTX on CTL proliferation and restimulatory capacity was studied in the context of the immunisation strategy outlined above, without tumour challenge. CTX was administered by low dose metronomic schedule at different times relative to MVAmel3 boost in order to observe the effect of timing of CTX treatment on numbers of $NP_{366-374}$-specific cells detected in the blood (Fig 3). For these analyses, total numbers of $NP_{366-374}$-specific cells in the blood were extrapolated from white blood cells counts, thereby taking into account the general reduction in numbers of white blood cells observed in CTX-treated animals. The effect of CTX on $NP_{366-374}$-specific responses was found to be dependent upon the timing of initiation of drug treatment. Thus, when CTX treatment was initiated on the same day as MVA boost, CTL proliferation was significantly impaired relative to immunised animals that did not receive CTX ($P = 0.043$, Student's t test), with total numbers of CTL at day 7 post-boost reduced by 90 %. Delaying drug treatment a further 7 days permitted initial CTL proliferation. However, these recently stimulated cells were also susceptible to CTX treatment, and numbers were rapidly reduced after drug treatment was initiated. Within 3 days of CTX treatment, the numbers of specific CTL were reduced by 92 % relative to immunised animals that did not receive CTX.

In contrast, when CTX was given to animals that had been primed and boosted 3 months previously, the levels of $NP_{366-374}$-specific CTL in the blood were not affected by drug treatment. Analysis of CTL in these animals prior to CTX treatment indicated that they expressed low levels of CD43 (Fig 3 A), a phenotype that has been associated with $CD8^+$ T cell memory. CTL in the recently activated groups expressed high levels of CD43, a phenotype associated with $CD8^+$ T cell 'effector phase'. Potent re-stimulatory capacity is a hallmark of T cell memory. To further investigate whether metronomic CTX treatment spares cells with a memory phenotype, each of the groups of animals above was subjected to a restimulation regime involving intravenous injection with syngeneic splenocytes that had been infected *ex vivo* with the recombinant vaccinia virus Vacc-mel3. This strategy was used because the previous MVA infection would have generated antibodies capable of neutralising a

second vaccinia virus infection. By administering splenocytes that had been infected *ex vivo*, these neutralising antibodies could be avoided temporarily, permitting presentation of the $NP_{366-374}$ peptide, and hence allowing CTL restimulation. CTX treatment was terminated 6 days before injection of infected splenocytes. Increases in numbers of $NP_{366-374}$-specific CTL following restimulation were observed in all animals, although this expansion was not statistically significant in animals that had received CTX during or soon after MVA boost (day 0 or day 7 after boost). In contrast, significant increases in $NP_{366-374}$-specific CTL were observed in those animals that were boosted over 90 days previously ($P = 0.036$), with restimulatory responses similar to control groups that were not treated with CTX ($P < 0.05$). These data suggest that CTX treatment causes reduction in the numbers of proliferating CTL, but does spare a cohort of cells with restimulatory capacity.

Example 5 – Combination of vinblastine and immunotherapy

To establish whether the combination therapy was effective when a different chemotherapeutic agent was used, the mel3-mediated immunisation strategy described in the previous examples was adopted, but combined with vinblastine, another drug shown to have anti-angiogenic qualities when delivered in a low dose metronomic fashion (Klement *et al. J. Clin. Invest* 2000; **105**:R15-24). DNA-mel3 immunisation and MVA-mel3 re-stimulation were performed as described in the above examples. B16-mel3 was administered one week after MVA-mel3 injection, and vinblastine therapy (1 mg/kg twice weekly) initiated on the same day. Once again, the combination of metronomic delivery of chemotherapeutic agent and immunotherapy induced the most powerful anti-tumour response (see Figure 4).

Example 6 – Combination of CTX and administration of dendritic cells

To establish whether the effective anti-tumour responses induced by combination of metronomic dosing of CTX and immunotherapy could be extended to other immunotherapeutic strategies, a model of immunotherapy utilising dendritic cells (DC) loaded with specific tumour antigen was adopted (Mayordomo *et al. Nat Med.* 1995 Dec; **1**(12):1297-302). In this experiment, dendritic cells that were cultured from bone marrow were loaded with the H-2 D^b-binding peptide from lymphocytic

choriomenigitis virus glycoprotein (LCMV $_{GP33}$) and administered *in vivo* by subcutaneous injection. Animals injected with DC that were not loaded with antigen served as controls. One week later, all animals were challenged with 3 x 10^5 B16 F10 melanoma cells that had been modified to express a minigene encoding the LCMV $_{GP33}$ peptide. For some of these animals ($n = 5$ per group), CTX therapy was initiated on the day of tumour challenge. The LCMV $_{GP33}$-specific immune response generated with the peptide-loaded DC provided significant protection against tumour challenge leading to a delay in tumour engraftment and growth. However, the combination therapy was, once again, the most powerful anti-tumour strategy, with complete protection from tumour challenge observed over the period of the experiment (see Figure 5).

Discussion

The examples show that the lowered toxicity of low dose metronomic drug treatment permits a degree of immune function, and hence that this treatment can be combined with immunotherapy. Specifically, it has been shown that metronomic dosing of CTX or vinblastine combines very effectively with anti-tumour immunotherapy administered by injection recombinant vectors encoding a tumour-specific antigen or by injection of dendritic cells dosed with tumour-specific antigen. This therapeutic combination can be applied to a variety of neoplasms for which tumour antigens have been defined.

While metronomomic administration of CTX caused significant reduction in CTL numbers in the blood immediately following initiation of drug treatment, the combination therapy was still dramatically enhanced over either treatment alone. Significantly, the combination of metronomic CTX and prime boost-mediated immunity was more potent than combination therapy using higher doses of the drug. This result highlights the importance of reducing CTX-induced cytoxicity directed at the immune effector cells. Supporting this suggestion, FACS analysis indicated that more CTL persist in the blood of animals receiving the low dose metronomic treatment compared to animals receiving the MTD. Interestingly, where metronomic dosing was used on immunised animals without tumour challenge, the CTL appear to

succumb to the CTX more rapidly (compare Figs. 2B and 3A). It is possible therefore that the presence of tumour may provide some CTL restimulation, thereby sustaining numbers in the blood for a longer period. Despite the detrimental effect of CTX on numbers of CTL, the combined anti-tumour activity of CTL and CTX was significant, implying that sufficient CTL survive to have impact.

Whilst not wishing to be bound by theory, it is possible that the efficacy of the combination therapy is a result of efficient CTL-mediated cytolysis in the first instance, which effectively reduces the tumour burden upon which the drug must act. Thus, while loss of antigen following immunotherapy alone results in unimpeded tumour growth, sustained CTX treatment is able to keep growth of the antigen-loss variants in check for a considerably enhanced period. Alternatively, the CTX treatment, whether by minimising angiogenesis, or by acting upon the tumour cells directly, may be preferentially removing proliferating tumour cells. The remaining cells, with a slower proliferative capacity, may take more time to provide antigen-loss variants that can avoid CTL-mediated deletion.

The doses of CTX used in the examples were taken directly from the report of Browder *et al, Cancer Res* 2000; **60**:1878-86, in which the antiangiogenic property of metronomic low dose CTX therapy was first reported. These experiments were also performed on C57BL/6 mice, with the metronomic dose provided as approximately one third of the MTD. It should be noted that some weight loss and discomfort was observed in animals that were subjected to metronomic dosing for periods in excess of 90 days. Thus it may still be desirable to lower the dose even further. A recent report has shown that CTX can be administered constantly in drinking water, effectively lowering CTX dose while sustaining antiangiogenic capacity (Man *et al. Cancer Res* 2002; **62**:2731-5). It is likely that such a strategy would result in less immune suppression, and less associated morbidity.

The effect of CTX on numbers of CTL in the blood appears to be dependent on the phenotype of these antigen-experienced cells. While even the low dose metronomic schedule induces a rapid reduction in numbers of recently activated cells, this

reduction is avoided if drug administration is delayed a period of months after MVA boost, by which time the majority of activated lymphocytes had reverted to a CD43 low phenotype. This phenotype has been described by (Harrington *et al, J Exp Med* 2000; **191**:1241-6 to be a memory phenotype. Our experiments show that these cells retain a significant restimulatory capacity, a hallmark of memory, regardless of whether they had been exposed to CTX or not (Fig. 3). CTX is a well-recognised alkylating agent, with its activity directed at the DNA of dividing cells. It is not surprising therefore that CTL in a proliferating "effector" phase fall prone to the activity of this drug, while cells activated months earlier, and approaching quiescence, do not.

The treatment of the present invention may be used, for example, where primary treatment either through surgical extirpation or radiation therapy has not been curative, or where the risks of developing subsequent metastatic disease are high; combination treatment could be administered in an adjuvant setting. Immunotherapy could be initiated before continuous metronomic low dose CTX or other chemotherapeutic administration to avoid the immunosuppressive properties of the drug. With the first signs of recurrent disease, immunotherapy by booster administration aimed at re-stimulating the tumour-specific memory compartment, can be effectively instituted during a temporary respite from metronomic dosing. Alternatively, in the setting of advanced local or metastatic disease, MTD chemotherapy could initially be at sufficiently high doses to debulk the tumour, followed by combination therapy as described.